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Preliminary studies on differential expression of auditory functional genes in the brain after repeated blast exposures

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Abstract—The mechanisms of central auditory processing involved in auditory/vestibular injuries and subsequent tinnitus and hearing loss in Active Duty servicemembers exposed to blast are not currently known. We analyzed the expression of hearing-related genes in different regions of the brain 6 h after repeated blast exposures in mice. Preliminary data showed that the expression of the deafness-related genes otoferlin and otoancorin was significantly changed in the hippocampus after blast exposures. Differential expression of cadherin and protocadherin genes, which are involved in hearing impairment, was observed in the hippocampus, cerebellum, frontal cortex, and midbrain after repeated blasts. A series of calcium-signaling genes that are known to be involved in auditory signal processing were also found to be significantly altered after repeated blast exposures. The hippocampus and midbrain showed significant increase in the gene expression of hearing loss-related antioxidant enzymes. Histopathology of the auditory cortex showed more significant injury in the inner layer compared to the outer layer. In summary, mice exposed to repeated blasts showed injury to the auditory cortex and significant alterations in multiple genes in the brain known to be involved in age- or noise-induced hearing impairment.

INTRODUCTION

Battlefield blast exposure is reported to cause auditory impairment in a large population of military personnel deployed to Iraq and Afghanistan [1–2]. Auditory/vestibular injuries from blast traumatic brain injury (TBI) can cause increased incidence of tinnitus and hearing loss, which worsens over time if not treated [1–4]. Shock waves generated from explosive blasts are reported to be destructive to both gas- and fluid-filled structures of the body, including the lungs, intestines, brain, eyes, nose, and middle ear [5–9]. Blast-induced damage to the auditory system can be the consequence of either direct exposure of the auditory canal to blast shock waves or TBI and impairment in the central auditory processing involving different brain regions after blast exposure. The literature on the neurobiological mechanisms of hearing impairment and development of tinnitus from blast TBI is limited.

Abbreviations: cDNA = complementary DNA, TBI = traumatic brain injury.

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A number of genes and their protein products have been reported to be involved in both age- and noise-related hearing loss [10–15]. Cadherin and protocadherin mutations were linked to digenic inheritance of deafness and have specific functional roles in noise-induced hearing loss [13–14,16–17]. Other groups of proteins involved in deafness are otoferlin and otoancorin, which are also reported to have major roles in auditory functions, including central auditory processing [18–21]. Another large class of molecules involved in auditory signaling is centered on the calcium regulating proteins, which are known to have broad functions in age- and noise-related hearing loss or protection [18,22–26]. The significance of reactive oxygen species and heat shock proteins in age- and noise-related auditory impairments are also reviewed in detail [10,27–34].

Recent research on age- or noise-related hearing loss preferred mice as a suitable animal model because of the vulnerability of mice to sound compared to other rodents [15,35]. We have developed a preclinical mouse model of repeated blast exposures using an air-blast shock tube that closely mimics the repeated exposure to improvised explosive devices, grenades, or firing weapons used in the battlefield or breacher's studies [36–37]. The newly developed repetitive blast animal TBI model showed significant levels of neuropathology and neurobehavioral deficits after repeated blast exposures at 20.6 psi [36]. Using this mouse model of repeated blast exposures, we sought to determine differential expression of auditory-related genes in various regions of the brain by complementary DNA (cDNA) microarray analysis.

METHODS

Animal Blast Exposure Model

Experiments were performed in male mice (C57BL/6J, age 8–10 weeks, Jackson Laboratory; Bar Harbor, Maine). Groups of isoflurane (4%) anesthetized animals ($n = 6$ for sham and $n = 6$ for blast) were exposed to repeated blast exposures (20.6 psi), as reported previously, using a shock tube [9,36,38–39]. At a 6 h time point after the last blast exposure, three animals each from sham and blast groups were euthanized, and the brain tissue was collected after necropsy and separated into various regions as described earlier [39]. Different regions of the brain samples were immediately snap frozen and stored at -80°C until use. Remaining animals ($n = 3$) in each group

were sacrificed at 24 h after the last blast exposure and used for histopathology.

Preparation of RNA

Total RNA was isolated using Trizol reagent (Invitrogen Life Technology; Carlsbad, California) following the manufacturer's protocol. RNA quality and quantity were determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, California).

cDNA Microarray Analysis

Microarray analysis was performed using Agilent 60-mer mouse genome 44K oligo microarrays (Agilent Technologies). We labeled 5 μg of purified RNA with a commercially available kit (Agilent Low Input Quick Amp) by polymerase chain reaction amplification (Bio-Rad Laboratories; Hercules, California). Samples were fragmented and hybridized against universal mouse reference RNA (Stratagene; La Jolla, California) with a kit from Agilent. A 2-Color Microarray-Based Gene Expression Analysis (version 6.5) protocol was used for labeling and microarray processing. An Agilent G2565CA fluorescence scanner was used to quantitate the slides, and the resultant data were extracted using software (Agilent Feature Extraction, version 10.7.1). For filtering and normalization of the data, GeneSpring 10.1 software (Agilent) was used.

Statistical Analysis

The statistical analysis of the microarray data was performed with GeneSpring 10.1 software. Changes in the level of expression of various genes after blast exposure in comparison to sham controls were identified by Welsh's *t*-test statistical method (*p*-values < 0.05) in conjunction with multiple correction test (Benjamini-Hochberg) with 5 percent false discovery rate. To account for the small sample size, we used the reference design and filtered for genes with signal intensities that are twice the standard deviation of the background intensity levels. We determined that by performing gene-by-gene *t*-tests, for a samples size of 3 and 5 percent false discovery rate and a standard deviation of 0.5, the power is 75 percent. We also applied pathway and gene ontology analyses that offer extra power because it is statistically unlikely that a larger fraction of false positive genes end up in one specific pathway.

Histopathology of Auditory Cortex

Histopathology was performed in blast-exposed and sham control mice ($n = 3$ in each group), as described previously [36]. Brain sections were silver stained and microscopically examined for neurodegeneration exclusively in the auditory cortex region, and the severity of injury was scored as mild (+), moderate (++) and severe (+++).

RESULTS

Expression of Auditory-Related Genes in Hippocampus After Repeated Blast Exposures

The hippocampus of mice exposed to repeated blasts showed significant changes in the expression of multiple genes that are reported to be involved in age- or noise-induced hearing loss (Table 1). Otoancorin, a gene defective in autosomal recessive deafness, showed a significant increase (3.4-fold), while otoferlin, which is essential for glutamate exocytosis at the auditory ribbon synapse, showed a 1.8-fold decrease in the expression after repeated blast exposures. The expression of calcium binding protein 2 showed a 1.6-fold increase, whereas calcitonin-related polypeptide expression showed a 1.9-fold decrease after

blast exposures. The expression of antioxidant enzyme superoxide dismutase 3 showed a 2.0-fold increase in the hippocampus of mice exposed to repeated blasts. The expression of heat shock protein 8 and heat shock transcription factor 5 showed significant increase in the hippocampus after repeated blast exposures. Protocadherin alpha 4 expression showed a 1.3-fold decrease after blast exposures.

Expression of Auditory-Related Genes in Cerebellum After Repeated Blast Exposures

The cerebellum of mice exposed to repeated blasts showed a 1.2-fold increase in protocadherins alpha 4 and beta 20 expression (Table 2). The expression of S100 calcium binding protein A7A showed a 1.4-fold increase, while multiple calcium channel proteins and calcium binding protein 2 expression showed a 1.1 to 1.2-fold decrease in the cerebellum after repeated blast exposures. Heat shock protein 8 expression also showed a 1.1-fold decrease after blast exposures.

Expression Profile of Auditory-Related Genes in Frontal Cortex After Repeated Blast Exposures

The expression of calcium signaling-related molecules showed significant increase in the frontal cortex of mice

Table 1.

List of auditory-related genes significantly altered in hippocampus after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Otoa	NM_139310	Otoancorin	+3.4	0.04
Cabp2	NM_013878	Calcium binding protein 2	+1.6	0.003
Sod3	NM_011435	Superoxide dismutase 3	+2.0	0.03
Hspb8	NM_030704	Heat shock protein 8	+1.3	0.02
Hsf5	NM_001045527	Heat shock transcription factor member 5	+1.8	0.02
Otof	NM_031875	Otoferlin	-1.8	0.04
Calca	NM_007587	Calcitonin/calcitonin-related polypeptide alpha	-1.9	0.04
Pcdha4	NM_007766	Protocadherin alpha 4	-1.3	0.001

Table 2.

List of auditory-related genes significantly altered in cerebellum after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Pcdhb20	NM_053145	Protocadherin beta 20	+1.2	0.03
Pcdha4	NM_007766	Protocadherin alpha 4	+1.2	0.03
S100a7a	NM_199422	S100 calcium binding protein A7A	+1.4	0.03
Cacng1	NM_007582	Calcium channel, voltage-dependent, gamma subunit 1	-1.2	0.003
Cacna2d1	NM_009784	Calcium channel, alpha 2, delta subunit 1	-1.1	0.049
Efcab2	NM_026626	EF-hand calcium binding domain 2	-1.1	0.01
LOC641192	XM_918536	Similar to heat shock protein 8	-1.1	0.046

exposed to repeated blasts, including calpain 3 (1.5-fold), S100 calcium binding protein A3 (1.4-fold), calcium/calmodulin-dependent protein kinase kinase 1 (1.2-fold), and calcium binding domain 4A alpha polypeptide 7 (1.4-fold) (**Table 3**). Protocadherin beta 11 and calreticulin expression showed significant decrease (2.2- and 1.2-fold, respectively) in the frontal cortex of mice exposed to repeated blasts.

Expression of Auditory-Related Genes in Midbrain After Repeated Blast Exposures

The changes in the expression of auditory-related genes in the midbrain of repeated blast-exposed mice are shown in **Table 4**. Expression of cadherin-like 24 showed a 1.8-fold increase, while expression of cadherin 12 and protocadherin 8 showed significant decrease (1.7- and 1.4-fold, respectively) after the blast exposures. Multiple calcium signaling molecules, including calpain 9 (2.1-fold), S100

calcium binding protein A3 (1.2-fold), and calcium activated potassium channel beta 3 (2.1-fold), showed significantly increased expression in the midbrain after repeated blast exposures. At the same time, the expression of calcium binding protein 7 (2.2-fold), calcium channel voltage dependent L type alpha 1D subunit (1.6-fold), and calcium/calmodulin-dependent protein kinase 2 gamma (1.1-fold) showed significant decrease after repeated blast exposures. The midbrain of repeated blast-exposed mice also showed significant decrease in the expression of heat shock protein 2 (1.3-fold), nicotinic alpha polypeptide 7 cholinergic receptor (1.5-fold), and stanniocalcin 2 (1.3-fold).

Histopathology of Auditory Cortex After Repeated Blast Exposures

To investigate whether blast exposure induces pathology of the auditory cortex, neuropathology analysis of the

Table 3.

List of auditory-related genes significantly altered in frontal cortex after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Capn3	NM_007601	Calpain 3	+1.5	0.03
S100a3	NM_011310	S100 calcium binding protein A3	+1.4	0.03
Camkk1	NM_018883	Calcium/calmodulin-dependent protein kinase kinase 1	+1.2	0.01
Efcab4a	NM_001025103	EF-hand calcium binding domain 4A alpha polypeptide 7	+1.4	0.04
Pcdhb11	NM_053136	Protocadherin beta 11	-2.2	0.004
Calr	NM_00759	Calreticulin	-1.2	0.04

Table 4.

List of auditory-related genes significantly altered in midbrain after repeated blast exposures in mice.

Gene Symbol	GenBank Accession No.	Gene Product	Fold Change	p-Value
Cdh24	NM_199470	Cadherin-like 24	+1.8	0.02
Capn9	NM_023709	Calpain 9	+2.1	0.03
S100a3	NM_011310	S100 calcium binding protein A3	+1.2	0.048
Gpx4	NM_008162	Glutathione peroxidase 4	+1.1	0.02
Ccs	NM_016892	Copper chaperone for superoxide dismutase	+1.1	0.03
Kcnmb3	NM_171828	Calcium activated potassium channel beta 3	+2.1	0.03
Pcdh8	NM_021543	Protocadherin 8	-1.4	0.02
Cadh12	NM_001008420	Cadherin 12	-1.7	0.01
Cabp7	NM_138948	Calcium binding protein 7	-2.2	0.04
Cacna1d	NM_028981	Calcium channel, voltage-dependent, L type, alpha 1D subunit	-1.6	0.03
Camk2g	NM_178597	Calcium/calmodulin-dependent protein kinase 2 gamma	-1.1	0.03
Hspb2	NM_178597	Heat shock protein 2	-1.3	0.04
Chrna7	NM_007390	Cholinergic receptor, nicotinic alpha polypeptide 7	-1.5	0.02
Stc2	NM_011491	Stanniocalcin 2	-1.3	0.04

brain of repeated blast-exposed mice was performed by silver staining. As shown in the **Figure**, a significant level of neurodegeneration occurred in the auditory cortex at 24 h after repeated blast exposures. The pathology index in the inner layer of auditory cortex (**Figure(b2)**) was scored as + to ++, while the pathology index of the outer layer (**Figure(a2)**) was – to + compared to the respective sham controls.

DISCUSSION

Previous studies showed a significant level of neuropathology and neurobehavioral changes, with ~20 percent mortality rate after repeated blast exposures in mice at 20.6 psi [36]. The pathology was more evident in the pre-frontal cortex and cerebellum of repeated blast-exposed mice. More recent results showed regional-specific changes in acetylcholinesterase activity in various regions of the brain after repeated blast exposures, indicating that the effects of blast exposure is heterogeneous in the brain [39]. The majority of the neurobiological changes in the brain were significant at 6 h after the last blast exposure [36]. Based on these observations, we analyzed the changes in the gene expression profile in different regions of the brain at 6 h after blast exposures in the present study.

The expression of otoferlin, which is known to be present in the brain and is essential for glutamate exocytosis at the auditory ribbon synapse and reported to be defective in a recessive form of human deafness, showed significant decrease in the hippocampus of mice exposed to repeated blasts [19–20,40–42]. In contrast, otoancorin, another hearing-related gene defective in autosomal recessive deafness and known to mediate the contact between the apical surface of sensory epithelial cells and acellular gels of the inner ear and the tectorial and otoconial membranes for proper auditory processing, showed significant increase in the hippocampus after repeated blast exposures [21,43]. Significant increase in the expression of otoancorin in the hippocampus after repeated blast exposures seems to be a compensatory mechanism to increase the sensitivity of hearing following injury to the auditory system and needs to be investigated in detail as a potential mechanism involved in the development of tinnitus.

Cadherins and protocadherins are another set of genes that showed differential expression in various regions of the brain after repeated blast exposures. Cadherin and protocadherin mutations are reported to be involved in noise-induced

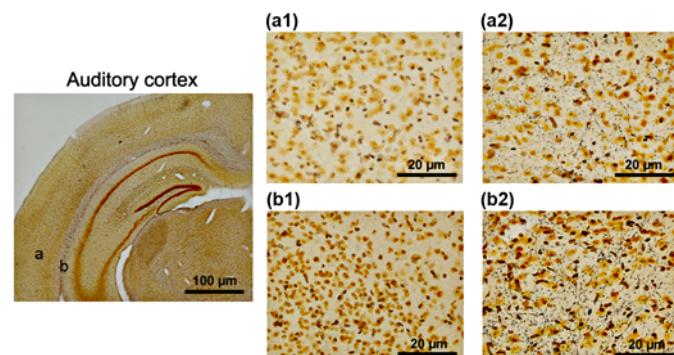


Figure.

Neuropathology of auditory cortex exposed to repeated blasts. Paraformaldehyde-fixed brain samples were sectioned into 50 μ m sections and stained with Neurosilver Kit II. Two different close proximities of auditory cortex indicated as **(a)** and **(b)** in left-most panel (labeled as “Auditory cortex”) were analyzed for severity of injury in sham control (**(a1)** and **(b1)**) and repeated blast-exposed (**(a2)** and **(b2)**) mice at 24 h after last blast exposure. Positive silver staining is evident in auditory cortex of repeated blast-exposed mice confirming neurodegeneration ($n = 3$ for sham and blast).

hearing loss [13–14,16–17,44]. The altered expression profile of cadherins and protocadherins in different regions of the brain after repeated blast exposures may impair central auditory processing. The functional significance of these gene modifications in the blast-induced impairment of central auditory processing has to be studied in detail to exploit them for therapeutic applications.

Molecules involved in calcium influx and calcium-dependent proteins/enzymes are predominant signal transducers in auditory neurons [22–23,45–48]. The frontal cortex and midbrain of blast-exposed mice showed significant increase in the expression of calcium-dependent cysteine proteases and calpain 3 and 9, respectively (**Tables 3** and **4**). Calpains are essential for initiation and promotion of cell death, and treatment with calpain inhibitors are known to prevent the hearing loss induced by aminoglycoside ototoxicity [45]. The hippocampus of blast-exposed mice showed a significant decrease in the expression of calcitonin-related peptide, a suggested peptide therapeutic treatment for hearing loss (**Table 1**) [22,48]. The cerebellum and midbrain regions showed significant decrease in the expression of voltage-dependent calcium channel genes after repeated blast exposures, while multiple calcium binding proteins showed differential expression in the hippocampus, cerebellum, frontal cortex, and midbrain after repeated blast

exposures (**Tables 1–4**). It is known that L-type voltage-gated calcium channels are involved in the pathogenesis of acoustic injury in the cochlea, and treatment with calcium channel blockers can reduce the damage to the auditory neurons [26].

Other types of molecules involved in calcium regulation, such as calreticulin and calmodulin-dependent protein kinase expression, showed significant decrease in the frontal cortex and midbrain of blast-exposed mice, respectively (**Tables 3 and 4**) [23,47]. Interestingly, two calcium binding proteins, calretinin and parvalbumin, that were upregulated in the cerebellum at 24 and 48 h after blast exposures by proteomic analysis were not found to be altered in cDNA microarray analysis at 6 h after blast exposures.* One possible reason for this difference is that calretinin and parvalbumin expression might be regulated at the translational level after blast exposures, which needs to be investigated further. Second, cDNA microarray analysis at 24 and 48 h after blast exposures needs to be done to find any significant changes in calretinin and parvalbumin gene expression. Western-blotting of the hippocampal region at 6 h after blast exposures showed significant increase in calretinin, further supporting the idea that blast exposure possibly modulates the protein expression at the translational level. The differential expression of calcium-dependent proteins/receptors in the brain after repeated blast exposures could be the consequence of increased/decreased calcium buffering in the auditory neurons. Thus, these results suggest that repeated blast exposures lead to an imbalance in the regulation of calcium homeostasis in different regions of the brain that can directly influence the central auditory processing and lead to auditory impairment.

Heat shock proteins or factors are one of the best-characterized families of protective proteins that are usually upregulated after stress, offering cellular protection and survival [10,27–28]. Repeated blast exposures in mice showed significant increase in the expression of heat shock protein 8 and factor 5 in the hippocampus, while cerebellum and midbrain showed significant decrease in heat shock protein 8 and heat shock protein 2, respectively (**Tables 1, 2, and 4**). The functional significance of heat shock proteins in hyper-

thermia and noise overstimulation is well documented [10,28]. The differential expression of heat shock proteins in the brains of repeated blast-exposed mice needs to be investigated further. Additionally, repeated blast exposure in mice showed significant reduction in the expression of the cholinergic receptor nicotinic alpha polypeptide 7 in the midbrain, suggesting a possible role of these receptors in aberrant central auditory processing (**Table 4**). The nicotinic receptor of cochlear hair cells has been proposed by others as a potential therapeutic target in acoustic trauma [11,49].

The functional role of reactive oxygen species and the protective efficacy of antioxidants in noise-induced hearing loss are well documented [32,50–51]. Repeated blast exposure in mice showed significant increase in the expression of antioxidant enzymes, superoxide dismutase 3, and glutathione peroxidase 4 in the hippocampus and midbrain, suggesting a protective mechanism in central auditory processing (**Tables 1 and 4**). The influence of glutathione peroxidase and superoxide dismutase in noise-induced hearing loss has also been reported [12,31,33–34]. Reactive oxygen species showed an increase in the brain following repeated blast exposures [36].

Neuropathology analysis of the auditory cortex of repeated blast-exposed mice showed significant injury (**Figure**). The injury level was more on the medial contralateral side of the brain than the ipsilateral side. The neuropathology of the auditory cortex is in line with the significant level of auditory-related gene expression changes in the brain of blast-exposed mice. It is not clear whether the neuropathology is responsible for the changes in gene expression or vice versa. It has been reported that blast-induced mild to moderate TBI leads to neurobiological and behavioral changes with multifocal axonal injury [52–53]. In these reports, neuropathological changes were observed at 7 and 14 d after blast exposure, although gene expression changes were observed at day 1, indicating that molecular changes contributes to the neuropathology. In our studies, neuropathology was prominent at 24 h after repeated blast exposures, but changes in gene expression were observed much earlier, suggesting that molecular changes can occur earlier as a direct effect of blast exposures. Preliminary data on brain DNA damage after blast exposure using comet assay showed breakage of DNA after repeated blast exposures. Studies with rats exposed to low-levels of explosive blast showed terminal dUTP nick end labeling-positive cells in the white matter in day 1 without any changes in day 7 [54].

*Arun P, Valiyaveettil M, Biggemann L, Alamneh Y, Wei Y, Oguntayo S, Wang Y, Long JB, Nambiar MP. Modulation of hearing related proteins in the brain and inner ear following repeated blast exposures. *Intervent Med Appl Sci.* Forthcoming.

Changes in many hearing-related genes after blast exposure in the brain indicate that these genes play specific roles in central auditory processing. The gene expression changes may be the consequence of initial protection against blast-induced central auditory processing and later as injury mechanism of central auditory processing. Gene expression changes can vary with respect to blast overpressure or number of blasts and may also depend on the severity of injury. The contribution of the shock waves transmitting through the auditory canal or directly through the skull in central auditory processing impairment is currently being investigated in the laboratory by using ear protection. Unraveling the functional role of these genes in central auditory processing and how they cross-talk with each of the brain regions to perform sound perception, hearing, speech recognition, and long-term memory will help us to understand how exactly their modulation plays a role in central auditory processing impairments. The linkage of these gene modulations to concurrent neuropsychiatric changes after blast exposure is also important to understand the complex neurobiological mechanisms of blast affecting central auditory processing and aid in rehabilitation.

CONCLUSIONS

In summary, preliminary results indicate that repeated blast exposures in mice showed significant alterations in multiple genes that are reported to be involved in age- or noise-related hearing loss at 6 h after blast exposure. The repeated blast exposure also showed significant neuropathology at 24 h in the auditory cortex, suggesting that blast exposure damages central auditory processing systems. Gene expression changes occur at early time points after blast exposure and may not be the consequence of apoptotic or necrotic changes in the brain. The gene expression profile showed differential pattern in various regions of the brain of mice exposed to repeated blasts. Otoferlin and otoancorin, which are involved in deafness, showed significant alteration in the hippocampus after repeated blast exposure. Similarly, cadherins and protocadherins, which are involved in noise-induced hearing loss, showed significant changes in all the brain regions tested. The expression profile of calcium-regulating proteins/receptors in various brain regions also showed differential expression, indicating an imbalance in calcium homeostasis after repeated blast exposures. The heat shock proteins and antioxidant enzyme expressions

also showed significant changes in various regions of the brain after repeated blast exposure, indicating possible protective effects. The differential expression of multiple auditory-related genes in various regions of the brain after repeated blast exposures in mice needs to be investigated further to draw specific biochemical pathways involved in the functional significance of central auditory processing in blast-induced auditory dysfunction and tinnitus.

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